A Method for high-frequency hetero-fusions of *Porphyra yezoensis* protoplasts with *Enteromorpha compressa* or *Monostroma nitidum* protoplasts

S. R. UPPALAPATI,[1)*] Teruo MORITA,[2)] and Yuji FUJITA[2)]

1) Graduate School of Marine Science and Engineering

2) Faculty of Fisheries, Nagasaki University, Bunkyo Machi 1-14, Nagasaki 852-8521, Japan

* To whom all correspondence should be addressed: fax: ++81 (0)95-844-3516; uppalapatisr76@hotmail.com

Low hetero-fusion frequencies between *Porphyra yezoensis* and *Enteromorpha compressa* or *P. yezoensis* and *Monostroma nitidum* protoplasts were observed when these protoplasts were fused following either of electrofusion or polyethylene glycol (PEG) mediated fusion methods. Pretreatment of protoplasts with actinase E (0.15%, 22℃ for 1 h) enhanced hetero-fusion frequency following both electrofusion and PEG methods. Maximum hetero-fusion frequencies were 7 - 8 % when *P. yezoensis* and *E. compressa* protoplasts were treated with actinase E and electrically fused. Electrofusion was optimally achieved when protoplasts were aligned at 25V (AC) for 20s and induced to fuse by a DC pulse of 250V for 25 μ s. Electrofusion method was found not suitable for hetero-fusions between *P. yezoensis* and *M. nitidum* protoplasts; hetero-fusion frequencies were low (1 - 2 %) although these protoplasts were treated with actinase E before electrofusion. Higher voltages to increase hetero-fusion frequency resulted in lysis of protoplasts. These difficulties were attributed to the differences in protoplast size of the fusion partners. PEG mediated fusion overcame these difficulties. Maximum hetero-fusion frequencies were 6 - 7 % when *P. yezoensis* and *M. nitidum* protoplasts were treated with actinase E prior to PEG mediated fusion.

Key Words: electrofusion, *Enteromorpha*, fusion frequency, hetero-fusion, *Monostroma*, polyethylene glycol, *Porphyra*, protoplasts

Introduction

Porphyra is one of the most important seaweeds in Japan. The genetic selection and improvement of this alga by means of conventional breeding techniques involving sexual crosses have not been very successful. Therefore intra-, inter-generic protoplast fusions are expected to be an effective breeding method in *Porphyra* improvement. In recent years many reports describing successful intra- and inter-generic protoplast fusions in *Porphyra* have been reported.[1)] Hybridization of distantly related species are well established in higher plants for breeding desirable hybrids.[2)] While the seaweed scenario of application of these techniques for genetic improvement is at its infancy. Wide hybridization involving fusions between phyla would extend the application of the protoplast fusion methods for breeding new seaweed hybrids. Hybrids with resistance to diseases (red rot and chytrid blight) and/or high amino acid contents with superior quality are desirable for commercial cultivation in Japan, to maintain the market value of 'Hoshi nori', with increasing overproduction in recent years.

But recently inter-phylum protoplast fusion between *P. yezoensis* and *Monostroma nitidum* and subsequent regeneration of the hybrid plants had been reported.[3)] Even though in seaweeds methods for high efficiency fusion of intra-generic protoplasts following electrofusion and as well as polyethylene glycol mediated fusion methods are well established, high hetero-fusion frequencies involving inter-phylum protoplasts are not reported (Table 1). A very low hetero-fusion frequencies of 1.4% were reported between *P. yezoensis* and *M. nitidum* protoplasts following PEG method.[3)] The survival rate of heterokaryons involving direct fusions in an inter-phylum protoplasts fusions are alarmingly low.[2,3)] Methods developed for achieving high hetero-fusion efficiency involving different fusion partners with *Porphyra* would be helpful in 1) widening the scope of introducing more genetic variability by fusion of *Porphyra* protoplasts with a variety of distantly related fusion parents, 2) increase the range of screening for desirable hybrids.

In this paper we report a method for high-frequency hetero-fusions between *P. yezoensis* protoplasts with *E. compressa* protoplasts following electrofusion method; and between *P. yezoensis* protoplasts with *M. nitidum* protoplasts following PEG mediated fusion.

Table 1. Comparison of optimum electrofusion parameters for the intra-and inter-generic & inter-phylum protoplast fusions in seaweeds

Fusion parents	Fusion parameters	% Hetro-fusion	Study
Porphyra yezoensis + *P. yezoensis* (green)	PEG 4000	12%	Fujita and Migita[4]
P. yezoensis + *P. yezoensis*	AC 40 V for 20 s DC 250 V for 40 μs	20%	Fujita and Saito[5]
P. yezoensis + *P. yezoensis* (green)	AC 40 V for 20 s DC 250 V for 40 μs	16%*1	Reddy *et al.*[6]
P. yezoensis + *P. yezoensis* (green)	3.0 kV/cm DC 300 V for 20μs	20%	Mizukami *et al.*[7]
Ulva pertusa + *U. conglobata*	AC 20 V for 10 s DC 200V for 20-25 μs	12%*1	Reddy *et al.*,[6] Reddy and Fujita[8]
U. pertusa + *Enteromorpha compressa*	AC 25 V for 10-15 s DC 250-300V for 25 μs	8-9%	Reddy and Fujita[9]
P. yezoensis + *E. compressa*	AC 25 V for 20 s DC 250 V for 25 μs	7-8%*2	Present study
P. yezoensis + *M. nitidum*	PEG 4000	1.4%	Kito *et al.*[3]
P. yezoensis + *M. nitidum*	PEG 4000	6-7%*2	Present study

*1 Protoplasts pretreated with protease P

*2 Protoplasts pretreated with actinase E

Materials and Methods

Vegitative thalli

Porphyra yezoensis Ueda (Bangiales, Rhodophyta) (T-14, a laboratory strain) foliose thalli were induced from conchocelis stock cultures maintained in the laboratory. *Enteromorpha compressa* (Linnaeus) Nees and *Monostroma nitidum* Wittrock (Ulvales, Cholorophyta) are collected from Oomura Bay, Nagasaki Prefecture, Japan and maintained as unialgal cultures in the laboratory. Young clean thalli were harvested and used for protoplast isolation.

Protoplast isolation

Isolation and purification of protoplasts from *P. yezoensis* were performed according to the methods described previou sly.[4] Protoplast isolation from *E. compressa* and *M. nitidum* was followed as reported by Reddy and Fujita,[9] these protoplasts were isolated by incubation in a filter-sterilized (0.22 μm, Millipore Ltd., USA) digestive solution, which was composed of 2% cellulase R-10, 1% macerozyme, 0.5% dextran sulfate, 0.6 M mannitol and 3.0 % NaCl in distilled water, and buffered with 50mM MES (2 [N-morpholino] ethane sulphonic acid) at pH 6.5. Thalli of *E. compressa* and *M. nitidum* were incubated in the digestive solution for 2 h at 20℃ in dark.

Protease treatment

In order to increase fusion frequency, isolated protoplasts were incubated in 2 % Protease P (Amano Pharmacy Co., Japan) for 20 min and 1.5% actinase E (Kaken Seiyaku Co., Ltd, Japan) for 1 h at 22℃. These protease solutions were prepared as reported by Fujita and Saito.[5] Actinase E solution was buffered at pH 7.5.

Electrofusion method

E. compressa protoplasts (Ec) were electrofused with isolated protoplasts from *P. yezoensis* (Py) in a somatic hybridizer SSH-2 (Shimidazu Corporation, Japan) using a FTC-02 (1 mm spacing) chamber. The protoplasts to be fused were suspended in the ratio of 1 : 1 in the fusion buffer, which was composed of 0.2M tris (hydroxymethyl) aminomethane, 1.0mM, $CaCl_2 \cdot 2H_2O$, 1.0 mM $MgCl_2 \cdot 6H_2O$ and 0.9M mannitol. To optimize fusion parameters these protoplasts were aligned under an AC current (10-30V) at 1 MHz applied for different duration (10-25s), with subsequent fusion following a DC pulse (150-300V) at a pulse width of 15-35 μs. The optimum parameters of AC current (25V) applied for 20s followed by subsequent fus ion at a DC pulse of 250 V for 25 μs are used in further fusion experiments. The hetero-fusion products (Py+Ec) were easily distinguished by their chloroplast pigmentation.

PEG fusion method

M. nitidum protoplasts (Mn) were induced to fuse with *P. yezoensis* protoplasts (Py) using polyethylene glycol (PEG 4000, Wako Chemicals Co. Ltd), according to the method of Fujita and Saito[5] using PEG 4000 powder. After eluting the PEG, the protoplats were collected by weak centrifugation (100 g for 4 min) and were resuspended in autoclaved modified f/2 medium.[10]

Hetero-fusion frequency

The hetero-fusion products of *P. yezoensis* and *E. compressa* (Py+Ec) and of *P. yezoensis* and *M. nitidum* (Py+Mn) were easily distinguished from unfused and/or homofusants by their distinct bi-color nature (green and red pigments). The hetero-fusion frequencies were calculated according to the following formula : Percent hetero-fusion frequency (%) = (number of hetero-fused protoplasts)/(total number of protoplasts)×100. The percentage of hetero-pairs and total protoplasts participated in alignment following AC current were calculated as : Percent hetero-pairs (%) = (number of protoplasts involved in hetero-pairs adhesions)/(total number of protoplasts involved in alignments in chains)×100. Total percent in chains = (total number of protoplasts involved in alignments in chains)/(total number of protoplasts)×100.

Results

Electrofusion method was adopted for hetero-fusion of *Porphyra yezoensis* and *Enteromorpha compressa* protoplasts. Hetero-fusion frequency without pretreatment with proteases was low, being at 1-2% (Fig. 1). Both protease P and actinase E pretreatment enhanced hetero-fusion frequencies and actinase E was more effective than protease P.

To further enhance the hetero-fusion frequency of the actinase E pretreated protoplasts, the electrofusion parameters were studied with actinase E treated *P.* yezoensis and *E. compressa* protoplasts. Electrofusion consisted of two steps. In the first step protoplasts were allowed to adhere with the other protoplasts in an AC field at 1 MHz. The optimum conditions for maximum percentage hetero-pairs were studied (Table 2). The application of low AC current applied for short durations yeilded low hetro-fusion pairing. An AC current applied for longer times has decreased the hetro-fusion pairs even though total protoplast involved in chains increased. The highest percentage of hetro-pairs of Py + Ec was 22% when an AC current of 25V was applied for 20s.

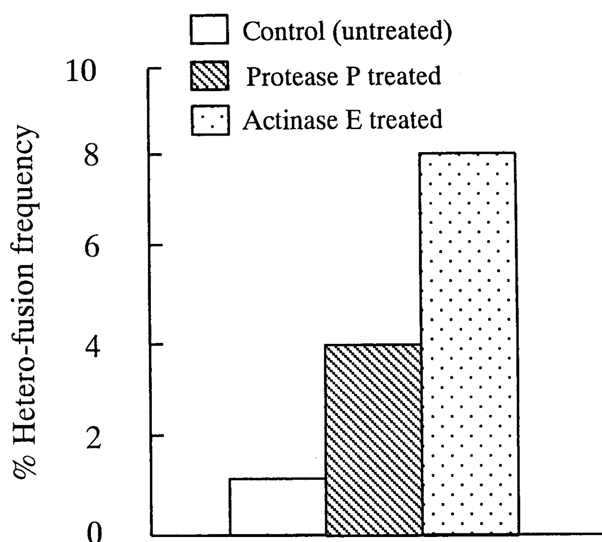

Fig. 1 Effect of protease P or actinase E treatment on hetero-funsion frequency of *Porphyra yezoensis* protoplasts with *Enteromorpha compressa* protoplasts (Py+Ec), following electrofusion method.

Table 2. Effects of AC voltage and applied time on percentage hetero-pairs (Py+Ec) of *Porphyra yezoensis* (Py) and *Enteromorpha compressa* (Ec) protoplansts

AC voltage (V)*1	Hetero-pairs % Py+Ec
10	4.0 (18.5)*2
15	6.5 (40.0)
20	12.0 (45.0)
25	21.5 (64.8)
30	9.4 (71.6)
Applied time (s)*3	
10	11.9 (33.0)
15	14.4 (40.3)
20	21.5 (64.8)
25	10.0 (70.0)

*1 Applied for 20s as fixed time
*2 Values in parenthesis is total % in chains
*3 The time is varied by fixing voltage at 25V AC

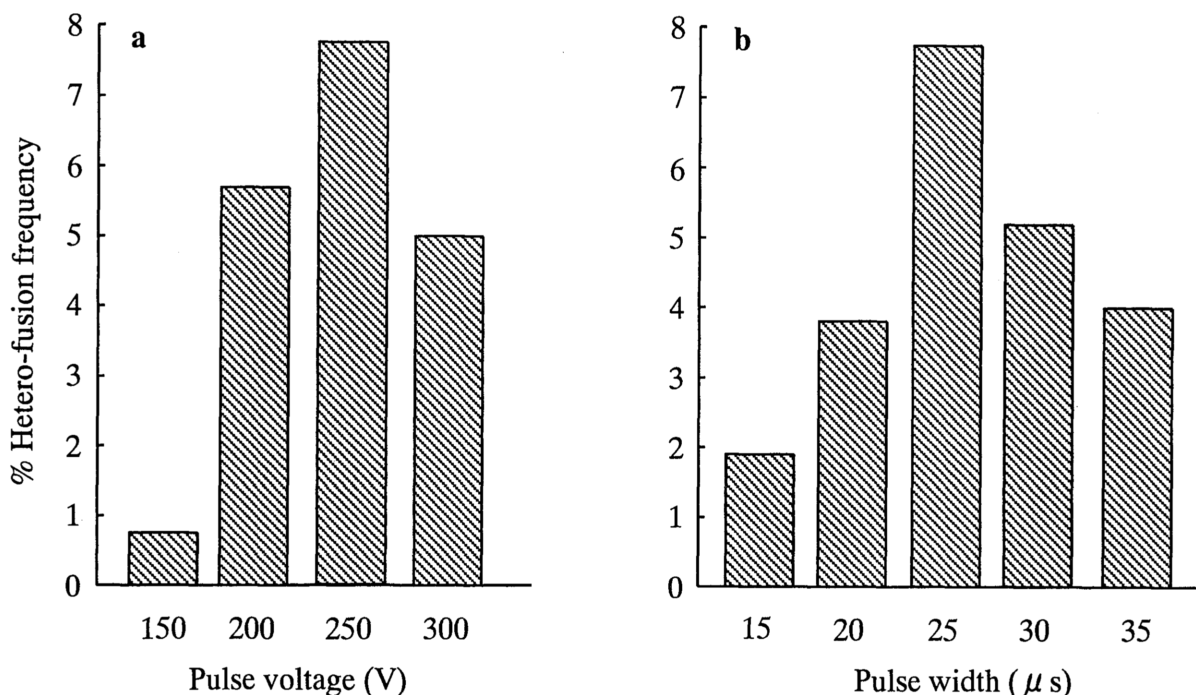

Fig. 2 Effect of DC pulse voltage (a) and applied pulse width (b) on hetero-fusion frequencies of *Porphyra yezoensis* protoplasts with *Enteromorpha* protoplansts (Py+Ec) following alignment at an AC current of 25V for 20s. The pulse width in (a) is 25μs and pulse voltage in (b) is 250V

The second step was induction protoplast fusion of the aligned protoplasts by the application of high intensity DC pulse of microsecond duration. The hetero-fusion frequencies of *P. yezoensis* and *E. compressa* as an interaction of pulse voltage and pulse width are shown in Figs. 2 a and b. Application of a DC current pulse of 250 V for a short duration of 25μs yielded maximum hetro-fusion products of and 7.8% (Py + Ec). When the DC pulse was applied for longer duration (>30μs), hetero-fusion products did not increase significantly, although the total fusion rates increased. Application of higher volatages (>300 V) for longer durations(>35μs) resulted in lysis of protoplasts.

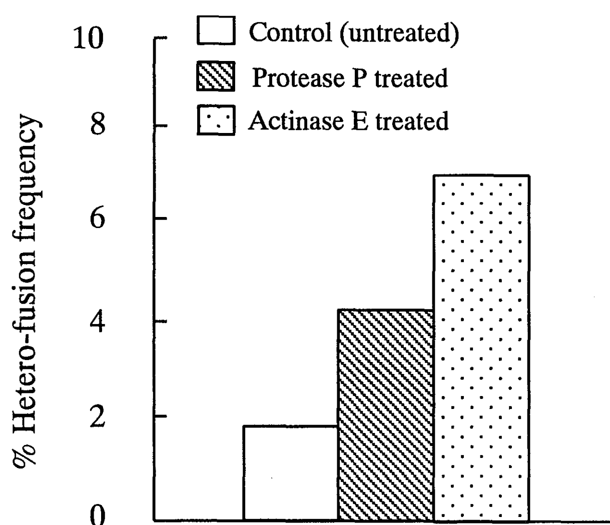

Fig. 3 Effect of protease P or actinase E treatment on hetero-fusion frequency of *Porphyra yezoensis* protoplasts with *Monostroma nitidum* protoplasts (Py+Mn), following PEG method.

Electrofusion methods yielded very low percent hetero-fusion frequencies (1 - 2 %) of *P. yezoensis* and *M. nitidum* protoplasts, and application higher voltages to enhance fusion frequency resulted in lysis of the protoplasts (data not shown). PEG mediated fusion was found suitable for hetero-fusion of *P. yezoensis* and *M. nitidum* protoplast s. However, fusion without protease P or actinase E pretreatment yielded a very low percent hetero-fusion frequencies of 1 - 2 %. An increase in hetero-fusion frequency was noticed following protease P treatment of the isolated protoplasts prior to the fusion. Hetero-fusion frequencies were further enhanced (4 - 5 %) by actinase E treatment of protoplasts (Fig. 3).

The hetero-fusion products obtained in the present study retained the bicolor nature even after 2 - 3 days facilitating easy isolation and culture of the hetero-fusion products (Fig. 4).

Discussion

Kameya[11] reported promoted fusion frequency of *Brassica* protoplasts by pronase E treatment. Reddy *et al.*[6] reported increased fusion rates of *Porphyra* and *Ulva* protoplasts by electrofusion of protease pretreated protoplasts. We have

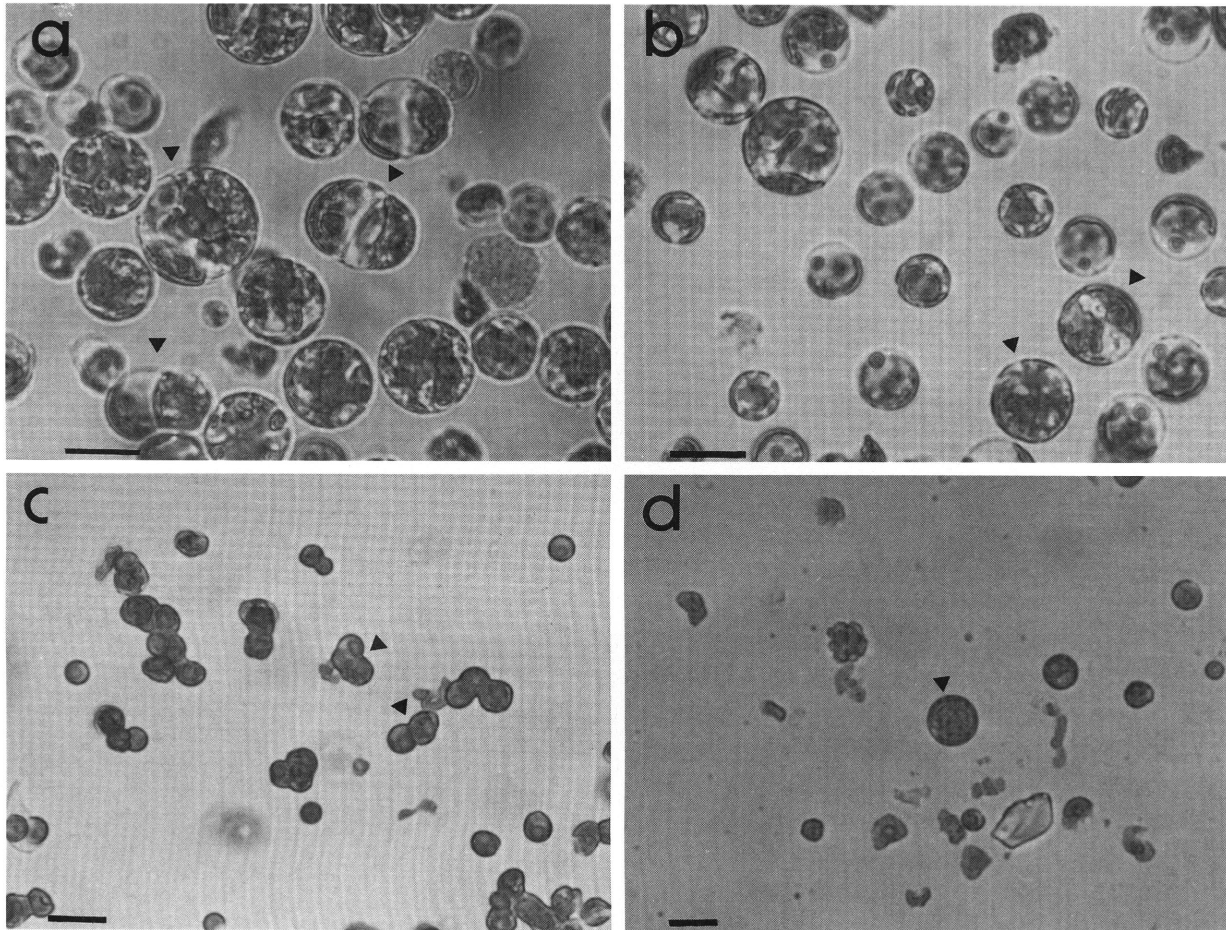

Fig. 4 Induction of high-frequency hetero-fusions of *Porphyra yezoensis* (Py) protoplasts respectively with isolated protoplasts from *Enteromorpha compressa* (Ec) and *Monostroma nitidum* (Mn)
a : Induction of electrofusion of Py and Ec protoplasts (arrowheads) (Scale bar=25 μ m)
b : Hetero-fusion products (Py+Ec) (arrowhead) after two days in culture (Scale bar=30 μ m)
c : PEG mediated fusion of Py and Mn protoplasts (arrowheads)
d : Hetero-fusion product (Py+Mn) (arrowhead) after three days in culture (c & d, Scale bar=30 μ m)

also observed increased fusion frequencies of protease P pretreated protoplasts, both through electrofusion and PEG methods. However actinase E was found to be more effective in the present study. The incompatibility of cell membrane proteins of the red and green algal membranes might be hindering higher fusion rates. Changes in membrane configuration might be facilitating fusion of protease treated protoplasts.[11,12] High hetero-fusion frequency following alignment at increasing AC current (25 V) observed, might be due to the pushing of the protoplasts against each other increasing the surface area of contact for fusion.[13] Increased fusion frequency were noticed for *P. yezoensis* and *E. compressa* protoplasts aligned at 25V (AC) through electrofusion. However the PEG method was found the most suitable for *P. yezoensis* and *M. nitidum* protoplast fusion. This may be attributed to the magnitude of difference in the protoplast radius of the fusion partners.[14]

The non-availability of genetic or biochemical markers makes it difficult to identify the reconstituted products at the early stage of development in seaweeds. The fusion of red and green protoplasts in the present study made the selection and isolation of putative hybrids easy. However in higher plants many chloroplast specific biochemical markers have been used for identification of the somatic hybrids.[15] Recently Kito et al. applied molecular analysis (RAPD) to confirm the nature of the putative hybrids.[3] These processes should further enhance the use and further application of protoplast fusion techniques for hybrid generation in *Porphyra*.

The high frequency fusion methods described in the present study along with the application of existing molecular techniques to confirm the hybrids can successfully be applied for introduction of genetic variability in *Porphyra*, for development of desirable strains for commercial 'nori' cultivation in Japan.

Acknowledgements

This work was partially supported by a grant in aid for Scientific Research from the Ministry of Education, Science and Culture, Japan.

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ヒラアオノリ及びヒトエグサのプロトプラストとスサビノリのプロトプラストとの高頻度異種融合の方法について

S.R.ウパラパティ，森田　晃央，藤田　雄二

スサビノリとヒラアオノリ，スサビノリとヒトエグサとの組み合わせで各々のプロトプラストを電気融合法またはポリエチレングリコール（PEG）法で融合したが，いずれの場合も融合の頻度は低かった。そこで，各プロトプラストをアクチナーゼE（0.15%，22℃，1時間）で前処理すると，電気融合法，PEG法のいずれの場合も異種融合頻度は高くなった。

アクチナーゼE処理したスサビノリとヒラアオノリとの電気融合は，最高値7～8％となった。この電気融合率の最適条件は，高周波電圧（AC）25V，20sの印加によってチェーンが形成され，パルス電圧（DC）250V，20μsの印加によって融合が誘導された。

電気融合法によるスサビノリとヒトエグサのプロトプラストの異種融合では，アクチナーゼEで前処理したプロトプラストの融合であっても，融合頻度は1～2％と低く，電気融合法が適さないことが分かった。異種融合の頻度を増すために，より高いパルス電圧をかけると，プロトプラストは破壊してしまった。これらの問題は，二種類のプロトプラストの大きさの違いによるものである。これらの問題を解決するために，スサビノリとヒトエグサとのあいだで，PEGによる異種融合を行うと，融合頻度は高くなった。アクチナーゼE処理したスサビノリとヒトエグサのプロトプラストの異種融合頻度は，PEG法によって最高値6～7％となった。